

Cell-Based Vital Organs Specific Biomarkers Assessment using Biofield Energy Based Novel Test Formulation

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Abstract

The aim of the present study was to determine the impact of Biofield Energy Treated test formulation using six different cell-lines. The test formulation/item (TI) and cell media (Med) was divided into two parts; one part was untreated (UT) and other part received Biofield Energy Treatment remotely by a renowned Biofield Energy Healer, Janice Patricia Kinney, USA and labeled as Biofield Energy Treated (BT) test item (TI)/media. Based on cell viability assay, test formulation was found as safe at tested concentrations. Cytoprotective activity of test formulation showed a significant restoration of cell viability by 60.6% (10 µg/mL), 67.5% (63.75 µg/mL), and 117.5% (63.75 µg/mL) in UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI, respectively compared to untreated in human cardiac fibroblasts cells (HCF) cells. Moreover, restoration of cell viability was improved by 64% and 127.3% in UT-Med + BT-TI and BT-Med + UT-TI, respectively at 1 µg/mL compared to untreated in human liver cancer (HepG2) cells. Cellular restoration in A549 cells was improved by 314% and 112.3% at 1 µg/mL in BT-Med + UT-TI and BT-Med + BT-TI, respectively than untreated. ALP activity in Ishikawa cells was significantly increased by 175.5%, 547.2%, and 220.8% in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI, respectively at 0.1 µg/mL as compared to untreated. Additionally, in MG-63 cells showed increased ALP activity by 76.9%, 78.4%, and 79% in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI, respectively at 50 µg/mL compared to untreated. The percent cellular protection of HCF (heart) cells (decreased of LDH activity) was significantly increased by 60.6% (10 µg/mL), 67.5% (63.75 µg/mL), and 117.5% (63.75 µg/mL) in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI, respectively as compared to untreated. An improved HepG2 cells protection (represents decreased ALT activity) by 115.1% (1 µg/mL), 42.5% (25.5 µg/mL), and 60.8% (10 µg/mL) in UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI, respectively as compared to untreated. Percentage cellular protection of A549 (lungs) cells (represents increased of SOD activity) was significantly increased by 191.1% and 81.4% at 0.1 µg/mL in UT-Med + BT-TI and BT-Med + BT-TI, respectively as compared to untreated. Serotonin level was significantly increased by 31.8% (10 µg/mL) and 56.9% (25.5 µg/mL) in UT-Med + BT-TI and BT-Med + BT-TI, respectively compared to untreated in human neuroblastoma cells (SH-SY5Y). Relative quantification (RQ) of vitamin D receptor (VDR) was significantly increased by 304.3% (0.01 µg/mL), 128.4% (0.1 µg/mL), and 240% (0.1 µg/mL) in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI, respectively compared to untreated in MG-63 cells. Thus, Biofield Energy Treated test formulation (The Trivedi Effect[®]) significantly improved organ specific functional biomarkers and would be useful for multiple organs health related to coronary artery disease, arrhythmias, congenital heart disease, cardiomyopathy, cirrhosis, liver cancer, hemochromatosis, asthma, chronic bronchitis, cystic fibrosis, osteoporosis, etc.

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Introduction

Herbs were one of the most prevalent therapeutic treatment and prophylactic approach in ancient times. However, vital nutrients such minerals and vitamins also play an important therapeutic value due to its huge medicinal purposes and its frequent use [1]. Medicinal plants, minerals, and vitamins are equally efficient to check and control the diseased state and imbalance during the pathological process. Thus, herbo-mineral formulations are one of the preferred choices of treatment as a Complementary and Alternative Medicine (CAM), as it contained specific metals or minerals as major composition, along with some vital herbs that have their beneficial effects on different biological systems. Herbo-mineral based drugs are now available in drug stores, food stores and also in supermarkets due to its high acceptance rates. More than four billion of the population about 80% living in developing world, they have accepted the herbal based drugs as one of the primary source of healthcare medical practice in various communities [2]. Therefore, in order to improve overall quality of life, the need of the hour is to formulate some novel drug formulation based on herbs, minerals and vitamins, which would be useful to improve health of the multiple organs. Biological processes are contributing a huge impact on human health, but the majority of the health issues are related with the multiple organ failure, which modulate ageing along with the risk of age-related frailty, disability, and associated diseases. Thus, mineral based test preparations are one of the preferred choices for overall health benefits as compared with phytomedicines. Thus, the novel test formulation was designed, which is composed of 11 important ingredients such as calcium chloride, magnesium gluconate, zinc chloride, sodium selenate, ferrous sulfate, vitamin B₁₂, vitamin D₃, ascorbic acid, vitamin B₆, panax ginseng extract, and beta carotene. This formulation is designed for overall

functioning of the organs that can results in improved overall health conditions against many pathological conditions such as lung disorder, liver disorder, breast cancer, liver cancer, aging, muscle damage, and overall health. Minerals and vitamins present in the test formulation provide significant functional support to all the vital organs [3-5]. In addition, panax ginseng is one of the best reported medicinal plants that improve mental, physical abilities, cognitive health, and is potent immunomodulator [6, 7]. The test formulation was tested against many cell lines and was evaluated for biological activities such as bone health parameters using MG-63 cells, lung health parameter using A549 cells, liver health parameter using HepG2 cells, heart health parameter using Human Cardiac fibroblasts, and neuronal health parameter using SH-SY5Y cells [8-17].

Biofield Energy Treatment was used as a CAM approach on test formulation and cell line based media through a renowned Biofield Energy Healer. Biofield Energy Healing Treatment has been reported with significant results in various clinical and preclinical studies with significant outcomes [18-20]. Biofield Energy is capable to suppression the mouse lung carcinoma growth along with anti-inflammatory activity [21]. Thus, Biofield Energy Healing therapies have gained significant worldwide popularity due to its improved immunological response [22]. CAM therapies have been recommended by National Center for Complementary/Alternative Medicine (NCCAM) and there therapies exist in various therapies such as external qigong, Johrei, Reiki, therapeutic touch, yoga, Qi Gong, polarity therapy, Tai Chi, pranic healing, deep breathing, chiropractic/osteopathic manipulation, guided imagery, meditation, massage, homeopathy, hypnotherapy, progressive relaxation, acupressure, acupuncture, special diets, relaxation techniques, Rolfing structural integration, healing touch, movement therapy, pilates, mindfulness, Ayurvedic medicine, traditional Chinese

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herbs and medicines in biological systems [23, 24]. The Trivedi Effect[®]-Consciousness Energy Healing therapies have been widely accepted and popular worldwide healing approach with significant results in many scientific field. The Trivedi Effect[®] has been reported with significant results in the metal physicochemical properties [25, 26], agriculture science [27], microbiology [28, 29], biotechnology [30, 31], improved bioavailability of many compounds [32, 33], skin health [34, 35], nutraceuticals [36], cancer science research [37], improved bone health [38-40], human health and wellness. Due to the continued clinical and preclinical applications of Biofield Energy Healing Treatments, the test formulation was studied for impact on the function of vital organs such as bones, heart, liver, lungs, and brain specific biomarkers in different cell-lines.

Materials and Methods

Chemicals and Reagents

Experimental chemicals were procured from standard company such as Panax ginseng extract was obtained from panacea Phytoextracts, India. Sodium selenate and ascorbic acid were obtained from Alfa Aesar, India. Silymarin and curcumin were obtained from Sanat Chemicals, India and quercetin was purchased from Clearsynth, India. Ferrous sulfate, vitamin B₆, vitamin D₃, vitamin B₁₂, calcium chloride, naringenin, trimetazidine (TMZ), 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT), and ethylenediaminetetraacetic acid (EDTA) were procured from Sigma Chemical Co. (St. Louis, MO). Zinc chloride, magnesium gluconate, β-carotene, and calcitriol were purchased from TCI chemicals, Japan. Reverse Transcription Kit, RNeasy Mini Kit, and Syber Green PCR kits were procured from Qiagen, India. All the other chemicals used in this experiment were analytical grade procured from India.

Biofield Energy Healing Treatment

The test formulation constituents and the specific cell line media was used for the treatment with the Biofield Energy. The test formulation was the combination of eleven ingredients such as panax ginseng extract, β-carotene, zinc chloride, calcium chloride, magnesium gluconate, sodium selenate, ferrous sulfate, ascorbic acid, vitamin B₁₂, vitamin D₃,

and vitamin B₆. The test formulation constituents and the cell line media were divided into two parts, one portion was considered as the untreated group, where no Biofield Energy Treatment was provided (UT-TI and UT-Med). Further, the untreated group was treated with a "sham" healer for comparison purposes, who did not have any knowledge about the Biofield Energy Healing Treatment. Another portion of the test formulation and the medium received the Biofield Energy Treatment (The Trivedi Effect[®]) remotely by Janice Patricia Kinney, under standard laboratory conditions for ~3 minutes through healer's unique Biofield Energy Transmission process and were referred as the Biofield Energy Treated formulation (BT-TI) and Biofield Energy Treated medium (BT-Med). The Biofield Energy Healer was located in the USA, however the test formulation constituents were located in the research laboratory of Dabur Research Foundation, New Delhi, India. Biofield Energy Healer in this experiment did not visit the laboratory, nor had any contact with the test sample and the medium. After that, the Biofield Energy Treated and untreated test items were kept in similar sealed conditions and used for the study as per the study plan.

Cell Viability Testing Using MTT Assay

All the experimental cells used in this study were counted for cell viability using hemocytometer in 96-well plates at the specific density as mentioned in the Table 1. The cells were then incubated overnight under standard growth conditions to allow cell recovery and exponential growth. Following overnight incubation, cells were treated with different concentrations of test formulations (BT/UT). After respective treatments, the cells were incubated in a CO₂ incubator at 37°C, 5% CO₂, and 95% humidity. After incubation, the plates were taken out and 20 μL of 5 mg/mL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to all the wells followed by additional incubation for 3 hours at 37°C. The supernatant was aspirated and 150 μL of DMSO was added to each well to dissolve formazan crystals. The absorbance of each well was read at 540 nm using Synergy HT microplate reader. The percentage cytotoxicity at each tested concentration was calculated using Equation 1:

$$\% \text{ Cytotoxicity} = [(R-X)/R] * 100 \dots \dots \dots (1)$$

Where, X = Absorbance of treated cells; R = Absorbance of untreated cells

Table 1. Information related to six cell lines with their plating density and time-point [41].

S. No.	Cell Line	Plating	Time Point
1	MG-63 (Bone)	3 X 10 ⁴ cells/ well, 96-well plate	5 days
2	Ishikawa (Uterus)	3 X 10 ⁴ cells/ well, 96-well plate	5 days
3	A549 (Lung)	10 X 10 ⁴ cells/ well, 96-well plate	24 hours
4	HepG2 (Liver)	1 X 10 ⁴ cells/ well, 96-well plate	24 hours
5	Human Cardiac fibroblasts (Heart)	1 X 10 ⁴ cells/ well, 96-well plate	24 hours
6	SH-SY5Y (Neuronal cell)	10 X 10 ⁴ cells/ well, 96-well plate	24 hours

The concentrations exhibiting percentage cytotoxicity <30% was considered as non-cytotoxic.

Cytoprotective Action of the Test Formulation

Cytoprotective effect of the test formulation in selected cells such as human cardiac fibroblasts-HCF; human hepatoma cells-HepG2; and adenocarcinomic human alveolar basal epithelial cells-A549 were counted and plated in suitable medium followed by overnight incubation. Further, the cells were then treated with the test items/positive control at the non-cytotoxic concentrations for 24 hours. After 24 hours, the oxidative stress using 10 mM t-BHP for 3.5 hours was given to the cells. The cells treated with 10 mM of t-BHP alone served as negative control. After 3.5 hours of incubation with t-BHP the above plates were taken out and cell viability was determined by MTT assay. The percentage protection corresponding to each treatment was calculated using equation 2:

$$\% \text{ Protection} = \frac{[(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{t-BHP}})] * 100}{[\text{Absorbance}_{\text{untreated}} - \text{Absorbance}_{\text{t-BHP}}]} \dots \dots \dots (2)$$

Estimation of Alkaline Phosphatase (ALP) Activity

For the estimation of ALP, two cells such as human bone osteosarcoma cells-MG-63 and human endometrial adenocarcinoma cells-Ishikawa were counted using a hemocytometer and plated in 24-well plates at the density corresponding to 1 X 10⁴ cells/well in phenol-free DMEM supplemented with 10% CD-FBS. After the respective treatments, the cells in the above plate were incubated for 48 hours in CO₂ incubator at 37°C, 5% CO₂, and 95% humidity. After 48 hours of incubation, the plates were taken out and processed for the measurement of ALP enzyme activity. The cells were

washed with 1 X PBS and lysed by freeze-thaw method *i.e.*, incubation at -80°C for 20 minutes followed by incubation at 37°C for 10 minutes. To the lysed cells, 50 μL of substrate solution *i.e.*, 5 mM of p-nitrophenyl phosphate (pNPP) in 1M diethanolamine and 0.24 mM magnesium chloride (MgCl₂) solution (pH 10.4) was added to all the wells followed by incubation for 1 hour at 37°C. The absorbance of the above solution was read at 405 nm using Synergy HT microplate reader (Biotek, USA). The absorbance values obtained were normalized with substrate blank (pNPP solution alone) absorbance values. The percentage increase in ALP enzyme activity with respect to the untreated cells (baseline group) was calculated using Equation 3:

$$\% \text{ Increase in ALP} = \frac{(X-R)}{R} * 100 \dots \dots \dots (3)$$

Where, X = Absorbance of cells corresponding to positive control and test groups

R = Absorbance of cells corresponding to baseline group (untreated cells)

Estimation of Lactate Dehydrogenase (LDH) in Human Cardiac Fibroblasts (HCF) Cells

HCF cells were used for the estimation of LDH activity. The cells were counted and plated at the density of 0.25 X 10⁶ cells/ well in 24-well plates in cardiac fibroblast specific medium followed by overnight incubation. The cells were then treated with the test formulation combinations/positive control at the non-cytotoxic concentrations for 24 hours. After 24 hours, oxidative stress was given to the cells using 10 mM t-BHP for 3.5 hours. The untreated cells were served as control group, which did not receive any

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treatment and were maintained in cell growth medium only. Cells treated with 10 mM of *t*-BHP alone served as the negative control. After 3.5 hours of incubation with *t*-BHP, the above plates were taken out and LDH activity was determined using LDH activity kit as per manufacturer's instructions. The percent increase in LDH activity was calculated using Equation 4.

$$\% \text{ Increase} = \frac{[(LDH \text{ activity}_{\text{sample}} - LDH \text{ activity}_{t\text{-BHP}})] * 100}{[LDH \text{ activity}_{\text{untreated}} - LDH \text{ activity}_{t\text{-BHP}}]} \dots \dots \dots (4)$$

Estimation of ALT in Liver Cells (HepG2)

The human hepatoma cells (HepG2) were used for the estimation of ALT activity. The cells were counted and plated at the density of 5 X 10⁴ cells/well in 48-well plates in DMEM media followed by overnight incubation. The cells were then treated with the test formulation/positive control at the non-cytotoxic concentrations for 24 hours. After 24 hours, oxidative stress was given to the cells using 400 μM *t*-BHP for 3.5 hours. The untreated cells served as control that did not receive any treatment and were maintained in cell growth medium only. Cells treated with 400 μM of *t*-BHP alone served as negative control. After 3.5 hours of incubation with *t*-BHP, the above plates were taken out and ALT activity was determined using ALT activity kit as per manufacturer's instructions. The percent increase in ALT activity was calculated using Equation 5.

$$\% \text{ Increase} = \frac{[(ALT \text{ activity}_{\text{sample}} - ALT \text{ activity}_{t\text{-BHP}})] * 100}{[ALT \text{ activity}_{\text{untreated}} - ALT \text{ activity}_{t\text{-BHP}}]} \dots \dots \dots (5)$$

Estimation of Superoxide Dismutase (SOD) in Lung (A549) Cells

The adenocarcinomic human alveolar basal epithelial cells (A549) were used for the estimation of SOD activity. The A549 cells were counted and plated at the density of 1 X 10⁴ cells/well in 24-well plates in DMEM followed by overnight incubation. The cells were then treated with the test formulation/positive control at the non-cytotoxic concentrations along with 100 μM *t*-BHP to induce oxidative stress. The untreated cells served as control that did not receive any treatment and were maintained in cell growth medium only. Cells treated with 100 μM of *t*-BHP alone served as negative control. After 24 hours of incubation with *t*-BHP the above plates were taken out and SOD activity was determined using SOD activity kit as per manufacturer's instructions. The percent increase in SOD activity was

calculated using equation 6:

$$\% \text{ Increase in SOD activity} = \frac{(X-R)}{R} * 100 \dots \dots \dots (6)$$

Where, X = SOD activity corresponding to test item or positive control

R = SOD activity corresponding to Control group.

Estimation of Serotonin in Neuronal Cells (SH-SY5Y)

The human neuroblastoma (SH-SY5Y) cells were used for the estimation of serotonin level. The cells were counted and plated at the density of 10 X 10⁴ cells/well in 96-well plates followed by overnight incubation. The cells were then treated with the test formulation/positive control at the non-cytotoxic concentrations. The untreated cells served as control that did not receive any treatment and were maintained in cell growth medium only. The treated cells were incubated for 24 hours. Serotonin release was determined by ELISA as per manufacturer's protocol. The percent increase in serotonin levels was calculated using equation 7-

$$\frac{(X-R)}{R} * 100 \dots \dots \dots (7)$$

Where, X = Serotonin levels corresponding to test item or positive control,

R = Serotonin levels corresponding to control group.

Effect of Test Formulation on Vitamin D Receptor (VDR) in Bone (MG-63) Cells

The effect of test formulation on vitamin D receptor (VDR) activity in bone (MG-63) cells were counted using the hemocytometer at density 2 X 10⁵ cells/well in 6-well plates followed by overnight incubation. The cells were then sera starved for 24 hours and treated with the test formulation/positive control at the non-cytotoxic concentrations, while control group did not receive any treatment, which were maintained in cell growth medium only. The treated cells were incubated for 24 hours and VDR expression was determined by qPCR using VDR specific primers. Cells were harvested by scrapping and washed with PBS. Cell pellets obtained were analyzed for VDR gene expression using human VDR specific primers: Forward: 5'-GCTGACCTGGTTCAGTTACAGCA-3', Reverse: 5'-CACGTCACTGACGCGGTA CTT-3'. VDR gene expression was normalized using House-keeping (HK) reference. Relative quantification (RQ) of VDR gene in Biofield Energy Treated cells was calculated with respect to the

untreated cells using equation 8:

$$RQ = 2^{-N} \dots \dots \dots (8)$$

Where, N is the relative Threshold Cycle (C_T) value of treated sample with respect to the untreated sample.

Statistical Analysis

All the experimental values were presented as percentage. The statistical analysis was performed using SigmaPlot statistical software (v11.0). For two group comparison, student's *t*-test was used. For multiple group comparison, one-way analysis of variance (ANOVA) was used followed by post-hoc analysis by Dunnett's test. Statistically significant values were set at the level of $p \leq 0.05$.

Results and Discussion

Cell Viability Using MTT Assay

All the cell lines such as MG-63, Ishikawa, A549, HepG2, HCF, and SH-SY5Y were screened for cell viability using MTT assay for safe concentrations. All the tested test concentrations of the test formulation were found safe on the basis of percentage of cell viability. The test criteria for non-cytotoxic test formulation concentration and the positive controls were found to be less than 30% cytotoxicity or greater than 70% cell viability. All the results were considered and represented as safe and non-cytotoxic concentrations. Overall, the

experimental data suggested that the overall percent cell viability in different cell-lines were found safe, which were tested for other activities.

Evaluation of Cytoprotective Effect of the Test Formulation

The test formulation was screened of cytoprotective activity against three cell lines *viz.* HCF, HepG2, and A549 cells, while the data was presented in terms of percentage cell protection against *t*-BHP induced cell damage (Figure 1). Trimetazidine (TMZ) was used as a positive control group in human cardiac fibroblasts cells (HCF) for cytoprotective effect which showed significant restoration of cell viability by 34%, 60%, and 98.3% at 5, 10, and 25 $\mu\text{g}/\text{mL}$, respectively as compared to the *t*-BHP induced group. Besides, the restoration of cell viability among the tested groups by the test formulation was reported as 60.6%, 46%, and 45.5% at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group; while, 67.5% improved cellular restoration at 63.75 $\mu\text{g}/\text{mL}$ in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 56.5% and 117.5% improved cellular restoration at 25.5 and 63.75 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + BT-TI group as compared to the untreated test group (UT-Med + UT-TI group). Similarly, silymarin was used as positive control in HepG2 cells, which resulted in significant

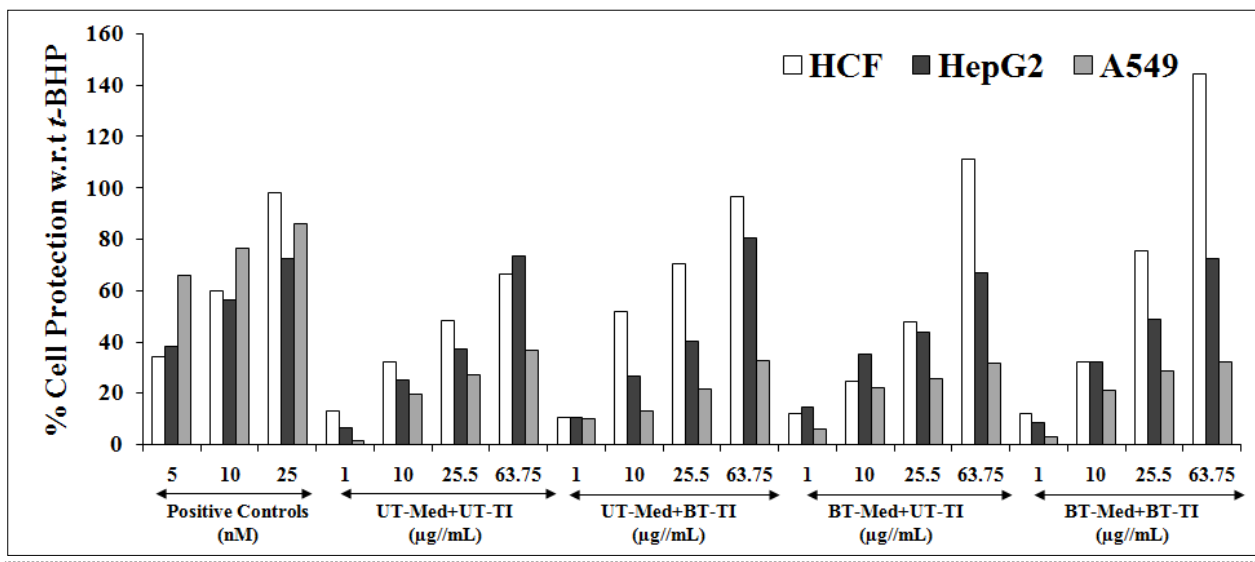


Figure 1. Cytoprotective action of the test formulation in human cardiac fibroblasts cells (HCF), human hepatoma cells (HepG2), and adenocarcinomic human alveolar basal epithelial cells (A549) against *tert* - butyl hydroperoxide (*t* -BHP) induced damage. Trimetazidine (μM), silymarin ($\mu\text{g}/\text{mL}$), and quercetin (μM) were used as positive control in HCF, HepG2, and A549 cells, respectively. UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

cellular restoration by 38.4%, 56.6%, and 72.6% at 5, 10, and 25 µg/mL, respectively as compared to the *t*-BHP induced group. Besides, the test formulation showed restoration of cell viability by 64%, 6.4%, 7.3%, and 10% at 1, 10, 25.5, and 63.75 µg/mL, respectively in the UT-Med + BT-TI group; while, 127.3%, 41.2%, and 16.6% improved cellular restoration at 1, 10, and 25.5 µg/mL respectively, in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 33.3%, 28.4%, and 30.4% improved cellular restoration was observed at 1, 10, and 25.5 µg/mL, respectively in the BT-Med + BT-TI group as compared to the untreated group. Additionally, quercetin was used as positive control in adenocarcinomic human alveolar basal epithelial cells (A549) resulted, restoration of cell viability by 65.7%, 76.7%, and 86.1% at 5, 10, and 25 µg/mL, respectively compared to the *t*-BHP induced group. Besides, the test formulation showed 605.3% restoration of cell viability at 1 µg/mL in the UT-Med + BT-TI group; while, 314% and 12.5% improved restoration of cell viability at 1 and 10 µg/mL, respectively in the BT-Med + UT-TI group as compared to the UT-Med + UT-TI group. Similarly, 112.3%, 6.5%, and 4.1% improved cellular restoration was reported at 1, 10, and 25.5 µg/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. These significant values states significant cytoprotective activity after oxidative stress using *tert*-butyl

hydroperoxide (*t*-BHP). However, this method has been considered as the gold standard for testing the cytoprotective action by stimulation in cell based assay [41, 42]. This cell based activity would reflects that the test formulation could be one of the best tool to protect the cells against injuries [43, 44], which could protect against many immune related disorders such as cardiovascular diseases, aging, cancer, diabetes, and many more [45-47]. Thus, Biofield Energy Treatment (The Trivedi Effect®) can be significantly used to protect the *t*-BHP induced oxidative stress against the HCF, HepG2, and A549 cells with respect to the cardiotoxicity, hepatotoxicity, and lung cell toxicity. Therefore, the Biofield Energy Healing Treatment could be used against many pathological etiologies such as cardiovascular, liver, and lung diseases.

Estimation of Alkaline Phosphatase (ALP) Activity

The test formulation and the test media was tested for ALP activity against two cell lines, MG-63 and Ishikawa cells after Biofield Energy Treatment. Naringenin (nM) was used as positive control in Ishikawa cells, and the results suggested significant increased ALP level by 9.5%, 23.7%, and 130.2% at 0.1, 1, and 10 nM respectively as shown in Figure 2. However, the experimental test groups showed an increased ALP activity by 175.5%, 72.1%, and 60.4% at 0.1, 10, and 50 µg/mL, respectively in the UT-Med + BT-TI group; while, 547.2%, 65.8%, and 30.3%

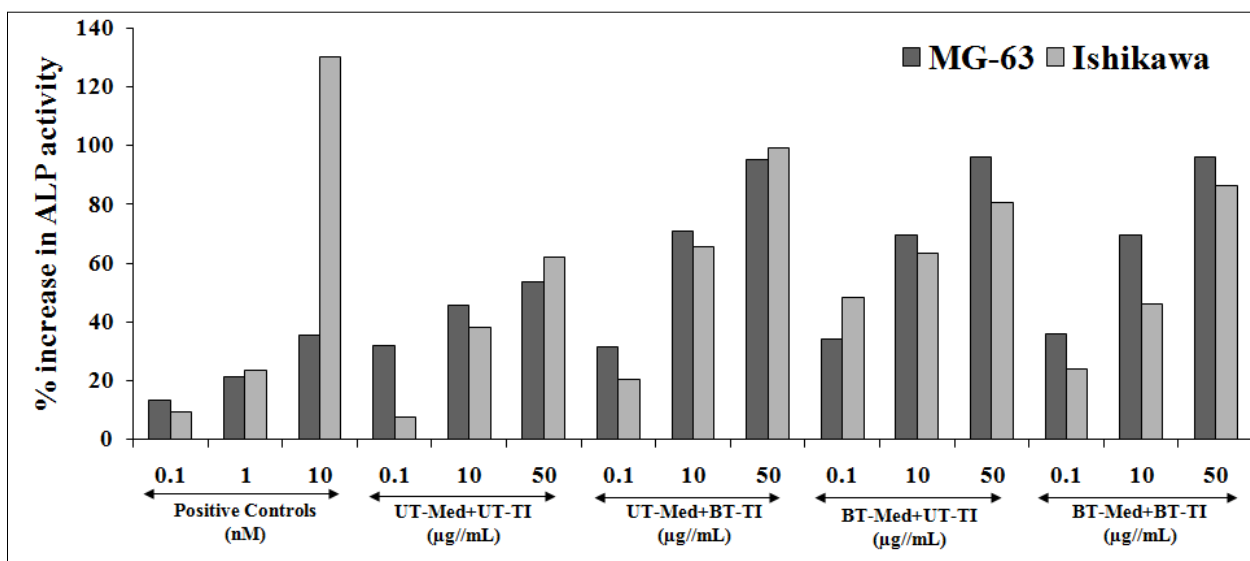


Figure 2. Alkaline phosphatase (ALP) activity in human bone osteosarcoma cells (MG-63) and human endometrial adenocarcinoma cells (Ishikawa) after treatment of the test formulation. Calcitriol and naringenin were used as positive control in MG-63 and Ishikawa cells, respectively. UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

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increased ALP activity at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 220.8%, 21.3%, and 39.4% improved ALP level was found at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group in Ishikawa cells. Similarly, calcitriol was used as positive control for MG-63 cells, and the data showed significant improved level of ALP by 13.2%, 21.4%, and 35.4% at 0.1, 1, and 10 nM, respectively. The ALP percent activity in MG-63 cells was significantly increased by 55.1% and 76.9% at 10 and 50 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Similarly, ALP percent was significantly increased by 6.8%, 51.4%, and 78.4% at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + UT-TI group as compared to the UT-Med + UT-TI group. However, ALP percent was significantly increased by 12%, 51.4%, and 79% at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group in the MG-63 cells. Thus, ALP level was significant improved after treatment with the Biofield Energy Healing Treatment. ALP is one of the important bone health biomarker responsible for controlling various bone disorders [48, 49] such as low bone density and osteoporosis, osteogenesis imperfect and Paget's disease, which makes bones brittle. Biofield Energy Treatment would be highly recommended option in

bone disorders without any adverse effects in comparison with the synthetic drugs.

Lactate Dehydrogenase (LDH) Activity in Human Cardiac Fibroblasts (HCF)

LDH activity was estimated in HCF cells, and the results after treatment in various experimental groups are presented in terms of improved HCF cellular protection, which represents decreased LDH activity in various groups. The effect of test formulation in different groups with respect to the percent protection of HCF cells in terms of decreased level of lactate dehydrogenase (LDH) activity is presented in the Figure 3. The positive control, trimetazidine (TMZ) showed 34%, 60%, and 98.3% increased cellular protection of HCF cells (decreased of LDH activity) at 5, 10, and 25 μM concentration as compared to the *t*-BHP group. The protection of HCF cells (decreased of LDH activity) was significantly increased by 60.6%, 46%, and 45.5% at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group; while, 67.5% improved cellular protection (decreased of LDH activity) at 63.75 $\mu\text{g}/\text{mL}$ in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 56.5% and 117.5% improved cellular protection (decreased of LDH activity) at 25.5 and 63.75 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Thus, the results suggested that significant

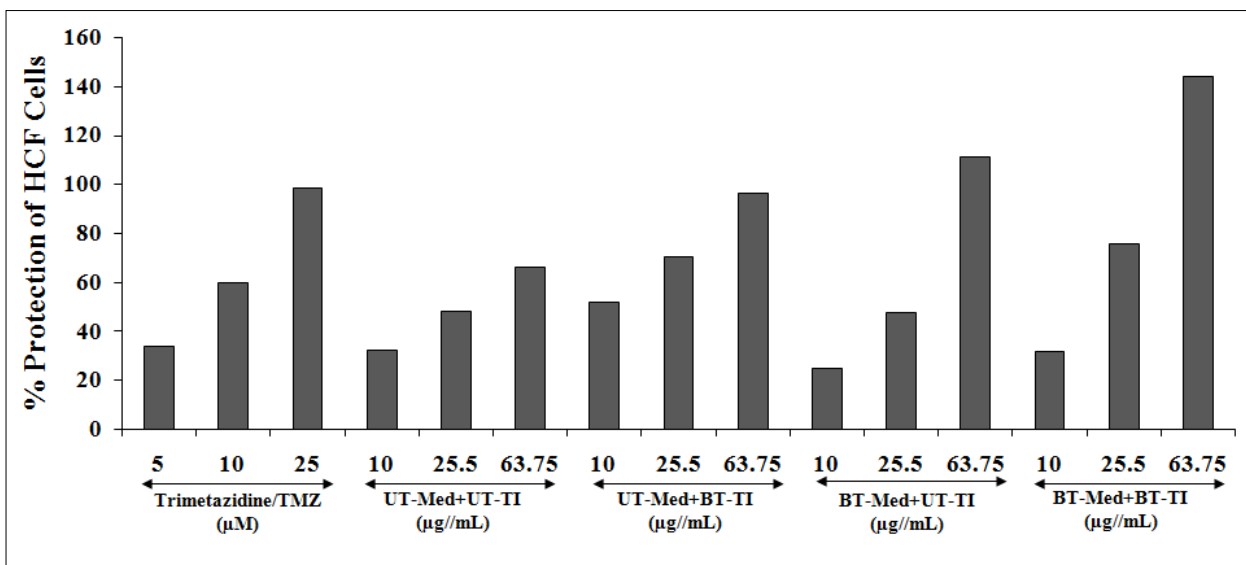


Figure 3. The effect of the test formulation on the increased percent protection of HCF cells, which represents decreased lactate dehydrogenase (LDH) activity against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. TMZ: Trimetazidine; UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

reduced level of LDH activity after Biofield Energy Healing Treatment. LDH activity using HCF cells was significantly reduction after Biofield Energy Treatment that could be useful against various pathological conditions such as tissue injury, necrosis, hypoxia, hemolysis or malignancies. LDH is an enzyme found in all the living cells and found to be responsible for anaerobic cellular respiration. LDH is extensively expressed in most of the body tissues, such as blood cells, skeletal muscle, and heart muscle and play a vital role in tissue injury, necrosis, hypoxia, hemolysis, or malignancies. Besides, LDH is the best biomarker for heart disease or tissue injuries. LDH activity can be best depicted using HCF cells, as these cells play a central role in the extracellular matrix maintenance of the normal heart along with synthesis of growth factors and cytokines [50-52].

Estimation of Alanine Amino Transferase (ALT) Activity in HepG2 Cells

ALT activity was estimated with the help of HepG2 cell and the results are presented in terms of increased percentage cellular protection (which represents decreased ALT activity) in the Figure 4. ALT is one of the important liver health enzymes along with kidney, heart, and muscles. Up and down regulation of this enzyme may results in hepatocellular injury and death [53]. The positive control, silymarin was in HepG2 cells for ALT activity and the data suggested increased percentage cellular protection of HepG2 cell (decreased

ALT activity) by 66.4%, 85.8%, and 114.4% at 5, 10, and 25 µg/mL, respectively. Similarly, the test formulation groups showed improved cellular protection of HepG2 cells (*i.e.*, decreased of ALT activity) by 115.1%, 22.7%, and 16.2% at 1, 10, and 25.5 µg/mL, respectively in the UT-Med + BT-TI group; while, increased cellular protection of HepG2 cells (decreased of ALT activity) by 12.8%, 16.5% and 42.5% at 1, 10, and 25.5 µg/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. An increased cellular protection of HepG2 cells (decreased of ALT activity) by 60.8% and 22.6% at 10 and 25.5 µg/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 4). Overall, the results showed significant activity after treatment with the Biofield Energy Healing Treatment. Hepatic cellular damage has been linked with high level of ALT, which affects the cell viability and damage to the cells [54]. Biofield Energy Treatment significantly improved the cellular protection with reduced ALT enzyme, which suggests its application in the liver cancer, liver cirrhosis, hepatomegaly, liver failure, and hepatitis.

Superoxide Dismutase (SOD) Activity in Adeno Carcinomic Human Alveolar Basal Epithelial Cells (A549)

SOD is one the best antioxidant defense mechanism of the body, which prevent the cellular damage against various types of stress and free radicals, which results in cell death [55]. SOD activity was

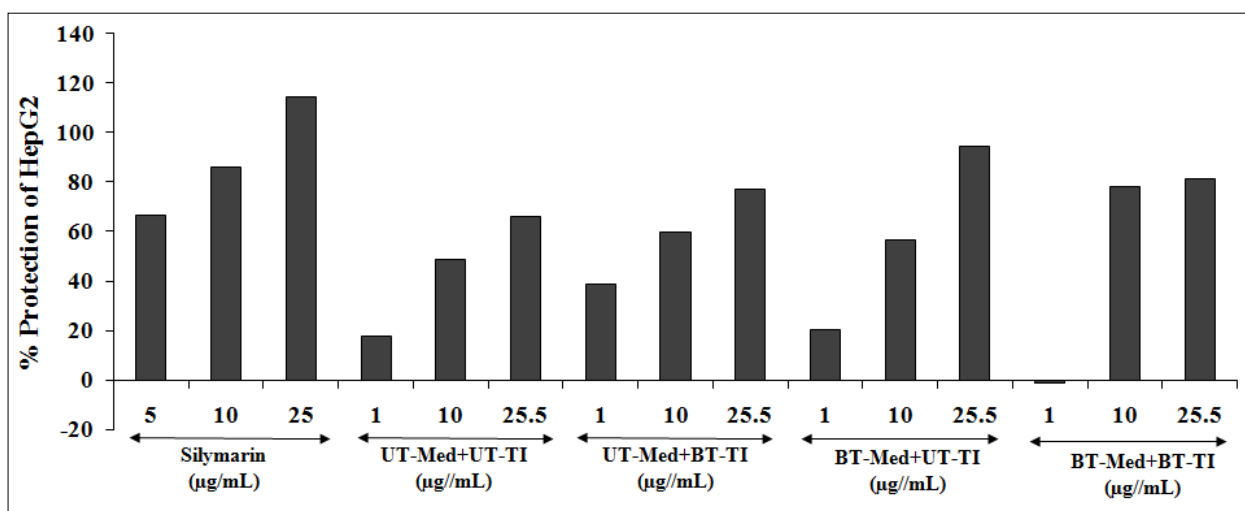


Figure 4. The increased percentage protection of liver cells (HepG2) that represents decreased (ALT) Alanine amino transaminase activity under the stimulation of *tert* - butyl hydroperoxide (*t* - BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

estimated using A549 cells and improved activity denoted increased cellular protection (Figure 5). The positive control, quercetin showed improved percentage increase in the SOD activity with respect to the *t*-BHP by 12.5%, 53.6%, and 62.1% at 0.1, 1, and 10 μ M, respectively. However, the percent protection of A549 (lungs) cells (increased of SOD activity) was significantly increased by 191.1%, 27.9%, and 47.4% at 0.1, 1, and 10 μ g/mL, respectively in the UT-Med + BT-TI group; while, increased SOD activity by 34.5% and 14.3% at 0.1 and 10 μ g/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover an increased SOD activity by 81.4% and 20.1% at 0.1 and 10 μ g/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 5). The present experimental data revealed that the Biofield Energy Treatment has significantly improved the SOD antioxidant defense activity, which could protect from many respiratory diseases such as pneumonia, asthma, pulmonary fibrosis, and lung cancer.

Estimation of Serotonin Level in Human Neuroblastoma (SH-SY5Y) Cells

Serotonin is supposed to be responsible for many neuropsychiatric disorders (*viz.* Alzheimer's disease, cognitive health, loss of ability of thinking,

depression, memory loss, etc.) along with various neuronal disorders like sleep, feeding, pain, sexual behavior, cardiac regulation, and cognition [56]. Serotonin assay was performed using SH-SY5Y cells and the effect of test formulation and cell line media was assessed after 24 hours of treatment using ELISA assay. Serotonin activity was tested and the data is presented in the Figure 6. Curcumin was used a positive control, showed 112.8%, 127.2%, and 160.2% increased the level of serotonin at 0.1, 1, and 5 μ M, respectively compared to the vehicle control (VC) group.

The data showed significant increased serotonin level by 31.8%, 17.6%, and 9.6% at 10, 25.5, and 63.75 μ g/mL, respectively in the UT-Med + BT-TI group; while, significantly increased serotonin level by 18.3% and 8% at 10 and 63.75 μ g/mL, respectively in the BT-Med + UT-TI group as compared to untreated group. Moreover, 56.9% and 40.2% improved serotonin level at 25.5 and 63.75 μ g/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 6). Overall, the present experiment showed that the serotonin level was significantly improved in the entire tested group. Our research study showed significant improved level of serotonin after treatment with the Biofield Energy Healing Treated test formulation that would be highly useful against various neurodegenerative diseases.

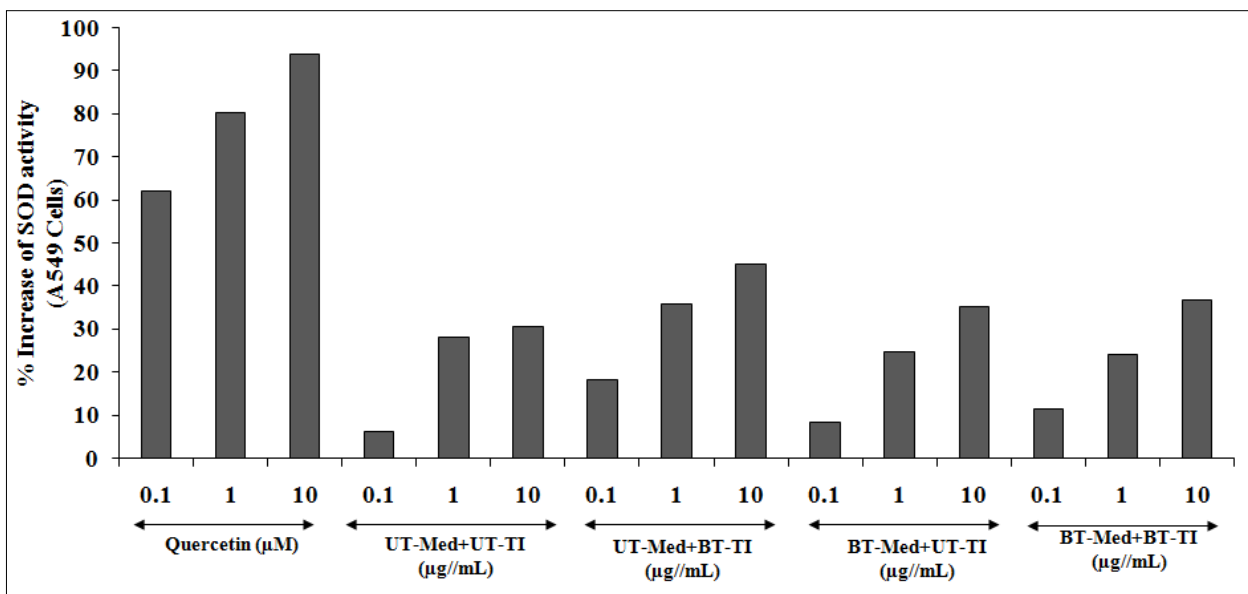


Figure 5. The improved percent protection of lungs cells (A549) in terms of increased SOD activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

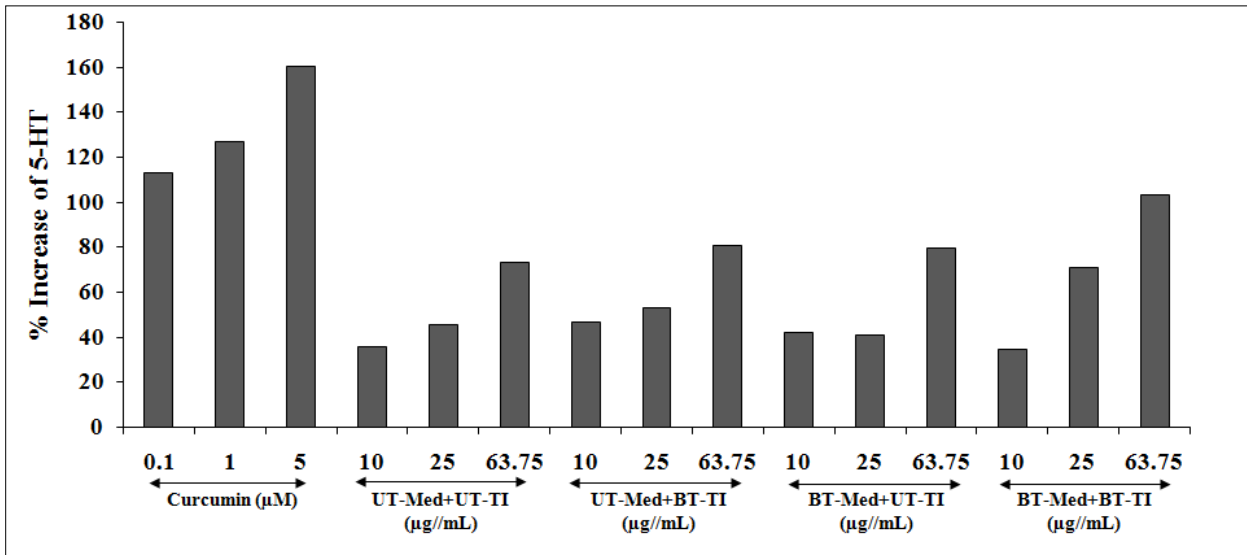


Figure 6. The effect of the test formulation on percent increase in 5-hydroxy tryptamine (5-HT) or serotonin in human neuroblastoma cells (SH-SY5Y). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item

Effect of Test Formulation on Vitamin D Receptors (VDRs)

Human bone osteosarcoma cells (MG-63) was used for evaluation of the VDR activity against the Biofield Energy Treated test formulation. The expression of VDRs was studied using the phenomenon of ligand binding through vitamin D active molecule, which was estimated using quantitative-polymerase chain reaction (qPCR) amplification. Using real time PCR, different VDR-relative threshold cycle (VDR-C_T) values were obtained after complete amplification cycles using specific primer probes. Relative quantification (RQ) was calculated from the VDR-C_T and house-keeping (HK)-C_T values in MG-63 cells. The values after treated with the Biofield Energy Treated and untreated test formulation and positive control are represented in the Figure 7. Calcitriol was used as a positive control and the RQ of VDR was found to be increased in concentration-dependent manner by 22.3%, 46.4%, and 171.3% at 1, 10, and 100 nM, respectively. The experimental test groups showed increased RQ of VDR expression by 304.3%, 277.7%, and 229.4% in the UT-Med + BT-TI group at 0.01, 0.1, and 1 µg/mL, respectively; while, 40.3%, 128.4%, and 86.4% increased RQ of VDR at 0.01, 0.1, and 1 µg/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover, an increased RQ of VDR by 240% and 221.8% at 0.1 and 1 µg/mL, respectively in the BT-Med + BT-TI group as compared

to the UT-Med + UT-TI group. Overall, it can be concluded from this activity, that VDR expression was significantly improved in MG-63 after treatment with the test formulation. Calcitriol was reported to bind with the VDRs and extensively regulates the calcium homeostasis, immunity, overall cellular growth, and differentiation [57]. Calcitriol controls various calcium metabolisms and play a vital role in improving quality of life and overall bone cell growth and development [58, 59]. The Trivedi Effect[®] would be the best alternative treatment approach for bone related disorders.

Conclusions

Cell viability (using MTT assay) data showed the test formulation was found safe and non-toxic against all the tested cell-lines. Cytoprotective activity against *t*-BHP induced cell damage was tested using human cardiac fibroblasts cells (HCF), which showed restoration of cell viability by 60.6% (10 µg/mL), 67.5% (63.75 µg/mL), and 117.5% (63.75 µg/mL) in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared to untreated. In HepG2 cells, the restoration of cell viability was observed as 64% and 10% at 1 and 63.75 µg/mL, respectively in the UT-Med + BT-TI group; however, 127.3%, 41.2%, and 16.6% improved cellular restoration at 1, 10, and 25 µg/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 33.3%, 28.4%, and

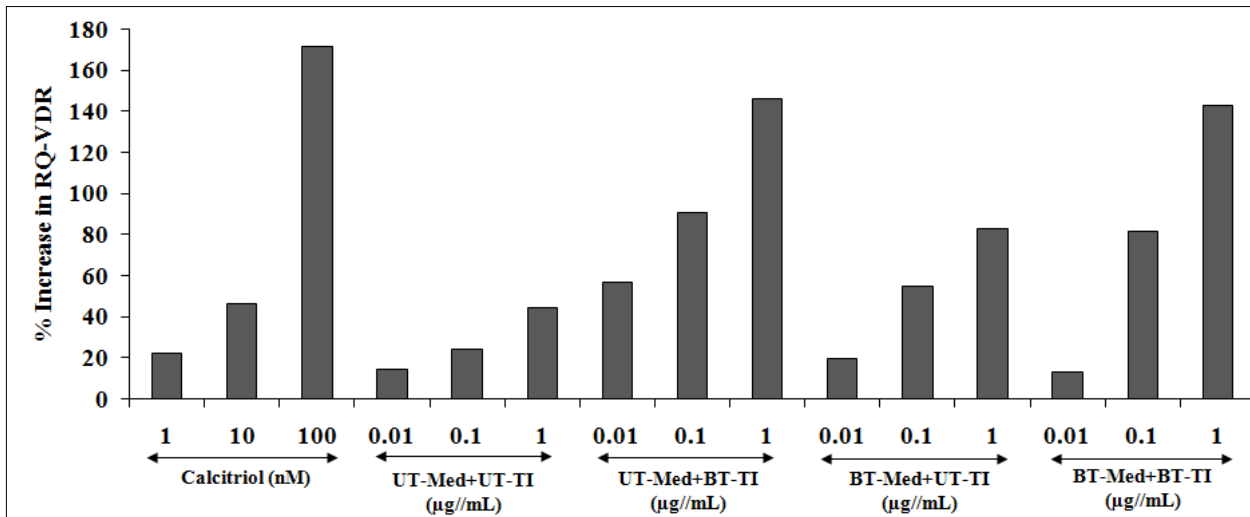


Figure 7. Relative quantification (RQ) of vitamin D receptors (VDRs) gene in human bone osteosarcoma cells (MG-63). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item

30.4% improved cellular restoration at 1, 10, and 25.5 µg/mL, respectively in the BT-Med + BT-TI group as compared to the untreated group. In A549 cells, cellular restoration was improved by 605.3% at 1 µg/mL in the UT-Med + BT-TI group; while, 314% and 12.5% at 1 and 10 µg/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 112.3%, 6.5%, and 4.1% improved cellular restoration was reported at 1, 10, and 25.5 µg/mL, respectively at BT-Med + BT-TI group as compared to the untreated test group. Similarly, ALP activity in Ishikawa cells showed significantly increased ALP activity by 175.5%, 547.2%, and 220.8% in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively at 0.1 µg/mL as compared to the UT-Med + UT-TI group. Similarly, ALP activity in MG-63 cells the cellular protection was reported by 76.9%, 78.4%, and 79% in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively at 50 µg/mL as compared with the untreated group. The percent protection of HCF cells (decreased of LDH activity) was significantly increased by 60.6% (10 µg/mL), 67.5% (63.75 µg/mL), and 117.5% (63.75 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared to the untreated group. ALT activity showed an improved cellular protection of HepG2 cells (decreased of ALT activity) by 115.1% (1 µg/mL), 42.5% (25.5 µg/mL), and 60.8% (10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared with the

untreated group. SOD activity was significantly increased by 191.1%, 34.5%, and 81.4% in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively at 0.1 µg/mL as compared with the untreated group. Serotonin level was significantly increased in SH-SY5Y cells by 31.8% (10 µg/mL), 18.3% (10 µg/mL), and 56.9% (25.5 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared with the untreated group. However, VDR expression was tested in MG-63 cells, which showed an increased RQ of VDR by 304.3%, 277.7%, and 229.4% at 0.01, 0.1, and 1 µg/mL, respectively in the UT-Med + BT-TI group; while, 40.3%, 128.4%, and 86.4% increased RQ of VDR at 0.01, 0.1, and 1 µg/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover, 240% and 221.8% increased RQ of VDR at 0.1 and 1 µg/mL, respectively in the BT-Med + BT-TI group as compared to the untreated group. Thus, this study concluded that the Biofield Energy based test formulation can improve the overall functioning of heart, liver, bones, neuronal, and lungs parameters against any oxidative stress or damage induced by free radicals. Biofield Energy Treatment (The Trivedi Effect®) can be used for the prevention of various types of cardiac disorders such as stroke, thromboembolic disease, congestive heart failure, congenital heart disease, peripheral artery disease, rheumatic heart disease, valvular heart disease, and venous thrombosis, etc. Besides, it would also protect against many hepatic

disorders (cirrhosis, liver cancer, hemochromatosis, and Wilson disease), lungs disorders (asthma, chronic bronchitis, emphysema, cystic fibrosis, and pneumonia), and many immune disorders. In addition, this novel test formulation can also be utilized for organ transplants (*i.e.*, kidney, liver, and heart transplants), hormonal imbalance, aging, and various inflammatory and immune-related disease conditions like Asthma, Aplastic Anemia, Graves' Disease, Dermatitis, Diabetes, Parkinson's Disease, Myasthenia Gravis, Ulcerative Colitis (UC), Atherosclerosis, etc. to improve overall health and Quality of Life.

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